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PRINCIPAL INVESTIGATOR: Barry W. Wilson, Ph.D.

CONTRACTING ORGANIZATION: University of California
Davis, California 95616-8671

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14. ABSTRACT Blood biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare agents and to establish whether some personnel are at greater risk than others from exposure. Many clinical and research laboratories use the colorimetric Ellman assay based on the hydrolysis of acetylthiocholine. CHPPM (US Army Center for Health Promotion and Preventive Medicine) uses a slower delta pH method based on that of Michel to monitor 16,000 DOD personnel each year. Two different approaches of ours yielded conversion factors for expressing delta pH AChE in terms of Ellman assay units. We also converted the normal range of AChE activities from the CHPPM delta pH assay to Ellman units generating important benchmarks for clinical laboratory determinations in the absence of baseline data. Future work includes determining conversion factors for the Test Mate cholinesterase measurements to the delta pH and Ellman methods, and examining the feasibility of monitoring serum BChE and PON1 activities in collaboration with the CRL laboratory of CHPPM.					
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INTRODUCTION

There is a need for rapid, high throughput, reliable and transferable determinations of blood cholinesterase (ChE) levels to provide early warning of exposures due to the intensive use of pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents. The colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al.*, 1961) is used by many clinical and research laboratories. A slower delta pH method, based on that of Michel (1949), is used to monitor the erythrocyte (RBC) acetylcholinesterase (AChE) of greater than 15,000 DOD personnel involved with chemical nerve agent and demilitarization operations each year. The assays are conducted by the Cholinesterase Reference Laboratory (CRL) at the US Army Center for Health Promotion and Preventive Medicine (CHPPM). Although pH assays are reliable and have low variability, they are not readily adaptable for kinetic analysis, automation or field use.

One goal of this project was to establish a conversion factor between the pH and colorimetric assays applicable to monitoring studies and field tests. Another goal was to provide conversion factors for the portable Test-Mate kit manufactured by EQM, Inc., purchased by the Army for “field use”. We showed that the current model is not adequate under “strict” field conditions (Oliviera *et al.*, 2002). Plans agreed to by the manufacturer were to produce a new model with improved assay parameters and adjustments.

Another issue is that of genetically sensitive individuals exposed to anticholinergic chemicals. Lowered plasma butyrylcholinesterase (BuChE), a scavenger of antiChE agents, may put individuals at increased risk to OP and CB agents (reviewed by Wilson, 1999). Paraoxonase (PON1) hydrolyses nerve agents (soman, sarin and VX) and the active oxon metabolites of widely used OP pesticides (diazinon and chlorpyrifos) in addition to paraoxon (Costa, *et al.*, 2005a). PON1 has been reported to be reduced in a cohort of veterans suffering from “Gulf War Syndrome” (Haley *et al.*, 1999). PON1 levels can also be modulated by lifestyle factors, such as diet, smoking and alcohol (Costa, *et al.*, 2005b). There is evidence that low levels of BChE and PON1 affect sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991). Following completion of the cholinesterase tasks, plans are to investigate the feasibility of screening for lowered BuChE and PON1 activities in CHPPM blood samples.

BODY

Materials

All chemicals were purchased from Sigma Chemical Co.

Methods

Sample collection

Blood was obtained from volunteers at UCD under an approved Human Subjects Protocol. Blood was collected in EDTA vacutainers and kept on ice. Within 4 hours of

collection, RBCs were separated by centrifugation at 1000 x g for 15 minutes and stored at 4°C.

Ellman Cholinesterase Assay

RBCs were diluted 1/50 in 0.5% Triton X-100, 0.1 M sodium phosphate buffer, pH 8. AChE activity was measured using a modified colorimetric method of Ellman *et al.* (1961) in 96 well plates at 25°C. The final concentrations of the substrate acetylthiocholine and the color reagent dithiobisnitrobenzoate (DTNB) were 1 and 10.3 mM respectively. Activity was reported as $\mu\text{mol}/\text{min}/\text{ml}$.

Delta pH Cholinesterase Assay

Delta pH measurements determined at CRL and UCD were performed according to Standard Operating Procedure # CRL40-2.7 provided by CRL. A 200 μl aliquot of RBCs was added to 4 ml of assay buffer (13 mM sodium barbital, 3 mM potassium phosphate monobasic, 510 mM sodium chloride, and 0.012% (w/v) saponin, pH 8.05). An initial pH measurement was recorded prior to adding acetylcholine bromide (10 mM final concentration), followed by a final pH measurement seventeen minutes later. The pH change of a substrate blank (no RBCs present) averaged 0.05 ± 0.02 delta pH/hr ($n = 9$). Assays were carried out at 25°C. Results were expressed as Delta pH/hour.

Task One. Conduct a careful comparison of the Ellman assay performed under optimum conditions and the DOD pH assay to examine the variability and reliability of both assays, to establish baseline values and to generate conversion factors to enable comparisons between them and other proposed or commercially available assays.

The main objectives of Task One have been completed. Human RBC AChE was measured with the CHPPM delta pH assay and the UCD Ellman assay, in split RBC samples collected by CHPPM. Linear regression analysis gives an estimated conversion factor between the two methods (Figure 1): $\text{Ellman} = 15.0(\text{delta pH/hr}) - 3.06$.

A second approach for comparing the two assay methods involved treating RBC preparations with varying concentrations of diisopropylfluorophosphate (DFP) to mimic OP-exposed samples. DFP is a well characterized AChE inhibitor commonly used in research. It is suited to *in vitro* tests because it does not require metabolic activation to inhibit AChE (as most OP pesticides do). Again regression analysis provides a conversion factor (Figure 2): $\text{Ellman} = 10.5(\text{delta pH/hr}) + 0.13$.

Earlier in the project, a normal range of delta pH ChEs was determined from 991 unexposed individuals in the DOD monitoring program, ranging from 18 to 76 years of age. There was no influence of age or gender. This data was converted into Ellman units by each of the two equations (Figures 3 and 4). The means and ranges of the converted CHPPM data are presented along with those of the initial UCD Ellman measurements in Table 1.

As proposed in the grant, we will complete this task by using a variety of OPs (XGB, diazinon-oxon, chlorpyrifos-oxon, paraoxon) to inhibit volunteer blood samples. These

will be assayed with both methods as a check of our conversion equations. The agents are on hand and facilities have complete UCD and USAMRICD approvals.

Before looking at conversions of the delta pH method to the Test-Mate kit (see Task 3), we have made a preliminary investigation of the linearity of the delta pH assay itself. This involves following the assay over time as a kinetic assay instead of the DOD protocol end-point measurement. The change in pH appears to be linear (Figure 5). We intend to investigate the kinetic linearity of the delta pH assay further.

Table 1. Ellman AChE activity of a population of DOD workers.

	n	Mean	S.D.	Min	Max
Direct UCD Assay	120	8.29	1.17	6.0	10.6
Interlaboratory Conversion	991	8.11	0.67	6.3	10.7
DFP Inhibition Conversion	991	7.98	0.65	6.7	9.2

AChE activity is umol/min/ml.

Min and Max represent the 95% limits of the population.

Task Two. Test the stability and usability of a red blood cell ghost standard suitable for clinical standardizations.

Task Two has been completed and the results have been published (Arrieta *et al.*, 2003). The activity of the earlier ghost RBC (gRBC) preparations was too low to measure with the delta pH method. We have increased the activity level in new preparations and are re-examining the issue. The ghost RBC standard is included routinely in each Ellman microplate assay conducted at UCD.

Task Three. Conduct experiments with a specially designed Test-Mate Kit with an uncorrected read out to establish the conditions for an optimum assay and construct conversion factors to harmonize its results with clinical laboratory assays.

Unfortunately, EQM president Pat Eberley says the new instrument has not been manufactured due to issues related to designing it to meet FDA approval. The Model 400 available commercially is the one we had studied in the past. We are still waiting for EQM to provide the next model for our collaborative study. Since the Model 400 is in use by the military, experiments to establish a conversion factor with the delta pH method are underway in collaboration with CHPPM.

Task Four. Explore the feasibility of incorporating BuChE variant and PON1 polymorphisms into a screen of workers for whom blood ChE baselines are required using a selected set of DOD personnel.

Currently, the conditions of blood collection performed by CHPPM are geared to RBC and not whole blood or plasma. We are discussing obtaining blood samples from CHPPM with its new director, Captain Gull and with the collaboration of Major Lefkowitz, the former manager of the facility. To do so will require modifying the collection procedures to permit us to examine the serum as well as RBC fractions.

One way to implement a screen for BChE and PON1 is to focus on activity levels of the two enzymes using butyrylthiocholine and the Ellman assay for BChE and the colorimetric two substrate (diazoxon and paraoxon) PON1 assay (Richter, *et al.*, 2004, Costa, *et al.*, 2005a). The equipment and facilities needed by CRL to perform the assays will be a consideration in designing a screening program. For example, blood cannot be collected in EDTA vacutainers for PON1 because the enzyme is calcium-dependent.

KEY RESEARCH ACCOMPLISHMENTS TO DATE

1. Established a normal range for human RBC AChE with CHPPM data.
2. Obtained two conversion factors for AChE activities between CHPPM delta pH data and Ellman values.
3. Used the conversion factors to generate a normal range of human RBC values in Ellman units, useful for clinical laboratories.
4. Demonstrated that the characteristic substrate inhibition curve for AChE is altered under conditions of the delta pH assay.

REPORTABLE OUTCOMES

Arrieta DE, SA McCurdy, JD Henderson, LJ Lefkowitz, RE Reitstetter and BW Wilson. 2004. Normal Range of RBC Cholinesterases in a DOD Monitoring Program. To be submitted.

Arrieta DE, VM Nihart, JD Henderson, RE Reitstetter, LJ Lefkowitz and BW Wilson. Comparing Cholinesterase Assays used to Detect Pesticide Exposure and Chemical Terrorism. Presented at the Annual National Institute for Occupational Safety and Health Meeting: "Cultivating a Sustainable Agricultural Workplace"; September 11-14, 2004; Troutdale, Oregon.

Arrieta DE, VM Nihart, JD Henderson, SA McCurdy, RE Reitstetter, LJ Lefkowitz and BW Wilson. Comparison of Delta pH and Ellman Colorimetric Cholinesterase Assays. Presented at the Bioscience 2004 Medical Defense Review; May 16-21, 2004; Hunt Valley, Maryland.

McCurdy SA, JD Henderson, DE Arrieta, LJ Lefkowitz, RE Reitstetter, and BW Wilson. Determining a Reference Value for Blood Cholinesterase using US Defense Department Personnel. Presented at the 43rd Annual Meeting of the Society of Toxicology; March 21-24, 2004; Baltimore, Maryland.

McCurdy SA, Henderson JD, Arrieta DE, Lefkowitz LJ, Reitstetter RE, Wilson BW. 2003. Normal range of cholinesterase levels among US Defense Department personnel. Presented at NIOSH Conference, November, 2003, San Francisco, CA.

Wilson BW, JD Henderson, DE Arrieta, SA McCurdy and RE Reitstetter. Conversion of Delta pH and Ellman Values for Cholinesterase. Presented at the 42nd Annual Meeting of the Society of Toxicology; March 9-13, 2003; Salt Lake City, Utah.

CONCLUSIONS

The Ellman assay is kinetic, whereas the delta pH assay is an endpoint determination. Our next step is to determine the activity rates for the delta pH method, making the delta pH test into a kinetic assay for the sake of the experiment.

The conversion values for delta pH and Ellman assays permit using the large CHPPM data base to establish a normal range for human RBC AChE in Ellman units, one of the goals of the project. Most clinical laboratories use assays based on the Ellman assay and rely either on unvalidated values listed in the commercial kits or their own records to establish a normal range to decide whether exposures have occurred in the absence of individual baseline data. For example the Roche kit lists 6.71-10.02 umol/min/mL for RBC AChE at 37°C and Yeary, *et al.* (1993) lists 7.86–12.9 umol/min/mL at 30°C. The range is expected to be higher when the assay is performed at a higher temperature, as with Yeary (30°C) compared to our range (25°C). The Roche range (37°C) is lower than might be expected, probably due to their suboptimal assay conditions which result in 40% lower activity (Wilson, *et al.*, 1995).

We are continuing to examine the Test-Mate kit. Underway is a study of the conversion of its results with the delta pH CRL method, in part, because of the continuing use of the Test-Mate kit by the Army as a field kit. We are using the current Model 400 kit at constant temperature as directed by the FDA approved instructions. The Army evaluation study of the Model 400 (Taylor *et al.*, 2003) used a mobile field laboratory with controlled temperatures but the Army's technical bulletin for the Test-Mate (available on the USAMRICD website) describes the use of the older OP Kit model in the outdoors rather than in the specified field laboratory setting (TB MED 296).

A research group at the WRAIR laboratory has developed a new ChE method (Feaster, *et al.*, 2001). We look forward to working with WRAIR and CHPPM on comparing this new method with the delta pH and Ellman assays, and how they can be made compatible with each other.

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APPENDICES

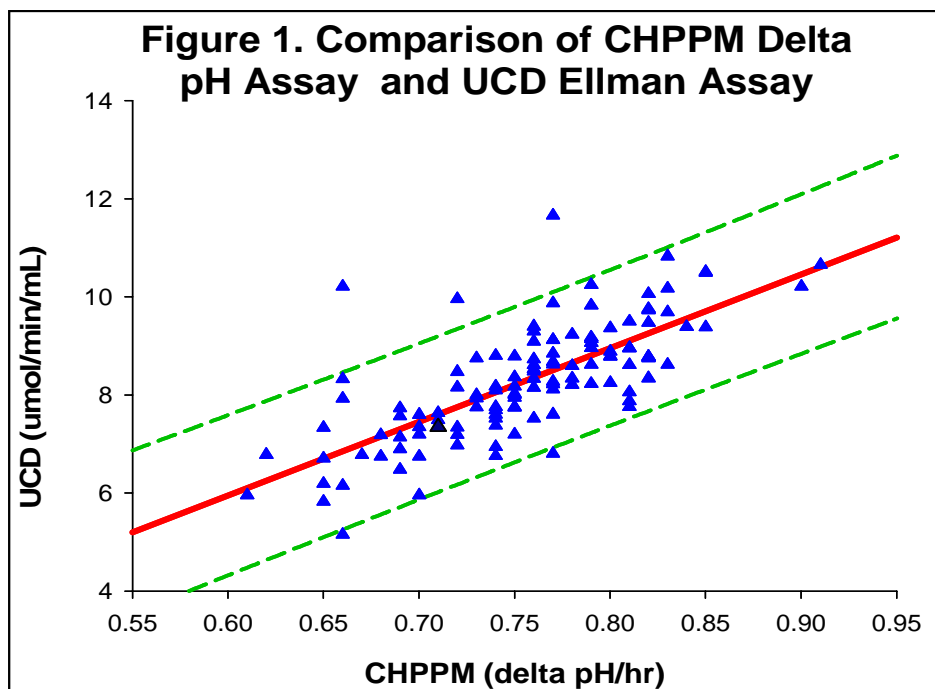
Figure 1. Comparison of CHPPM Delta pH Assay and UCD Ellman Assay

Figure 2. Comparison of ChE Methods Using DFP-Treated Human Blood

Figure 3. RBC AChE Distribution: Interlaboratory Equation Conversion

Figure 4. RBC AChE Distribution: DFP Inhibition Equation Conversion

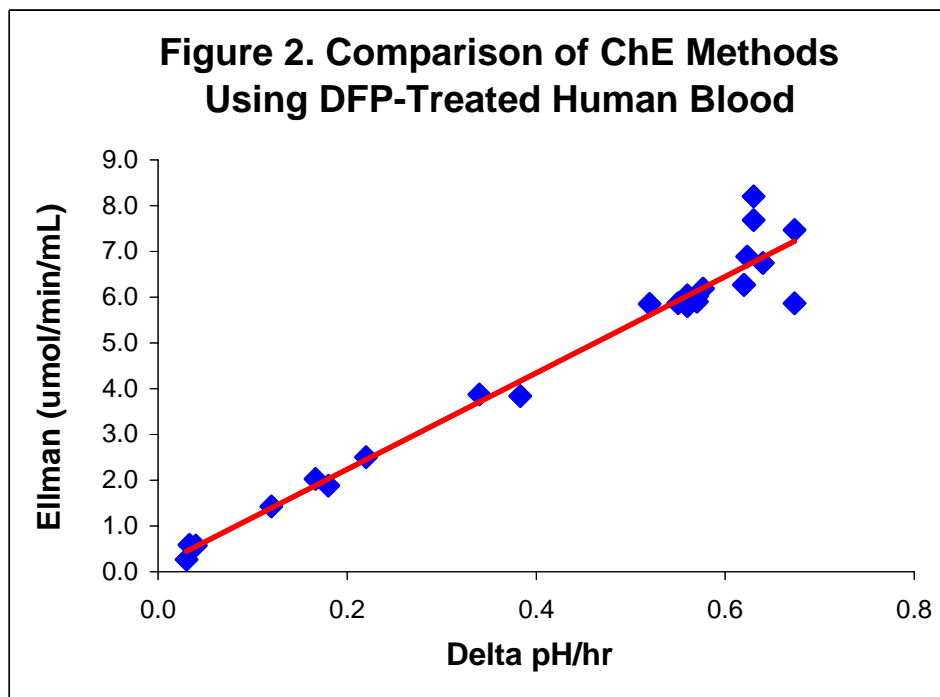
Figure 5. Kinetic Measurement of the Delta pH Assay



Split aliquots of human RBC samples assayed at UCD and CRL.

$n = 120$; $r^2 = 0.53$

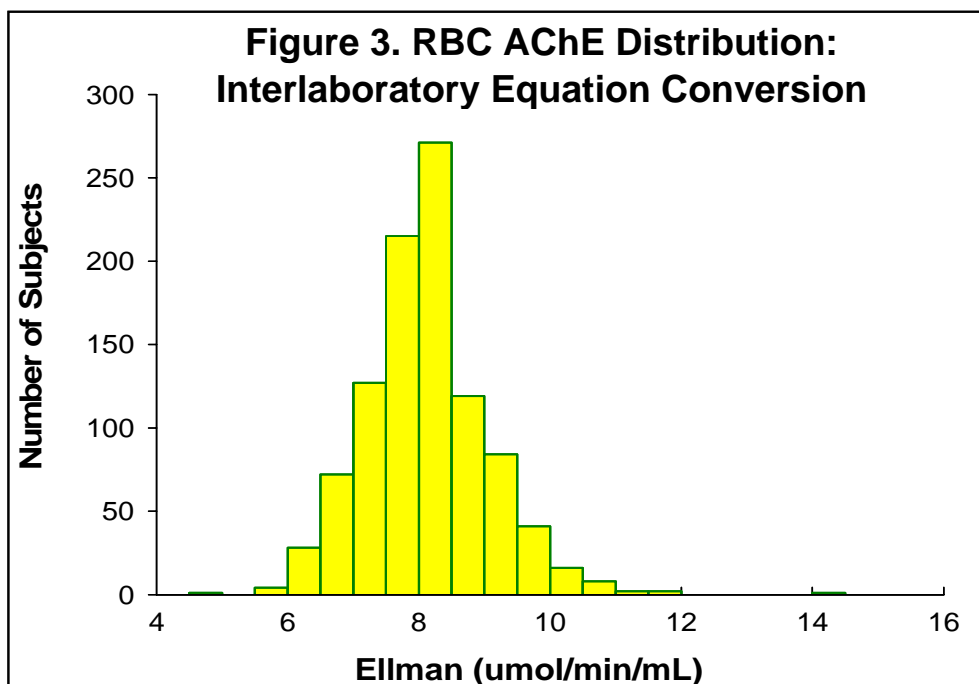
Line equation: Ellman = $15.0(\text{delta pH/hr}) - 3.06$



Human RBCs treated with varying concentrations of DFP assayed at UCD.

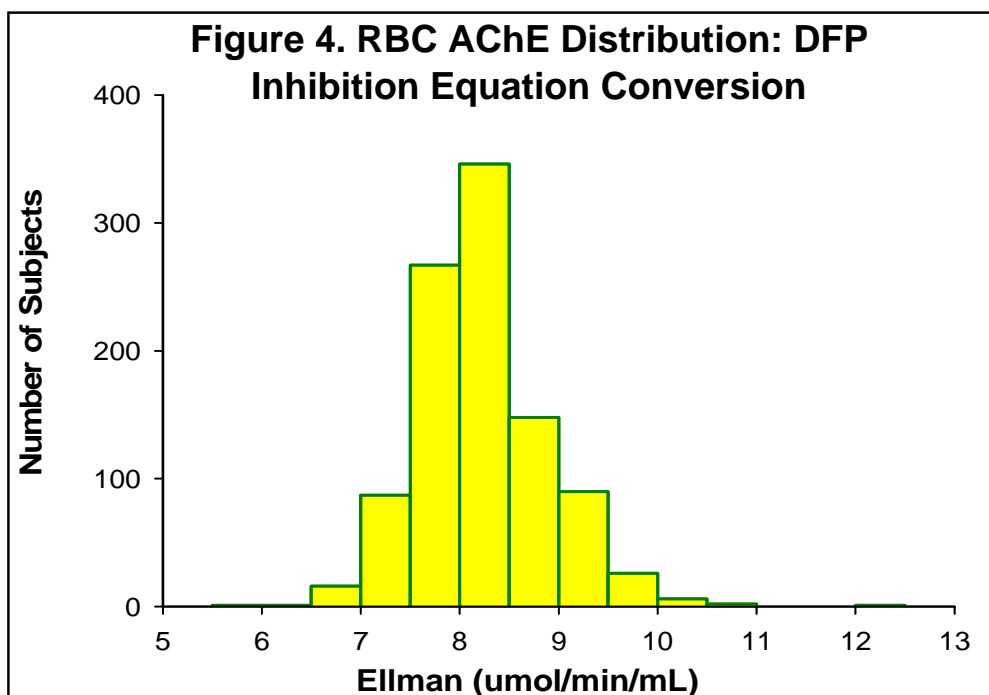
$n = 3$; $r^2 = 0.96$

Line equation: Ellman = $10.53(\text{delta pH/hr}) + 0.13$



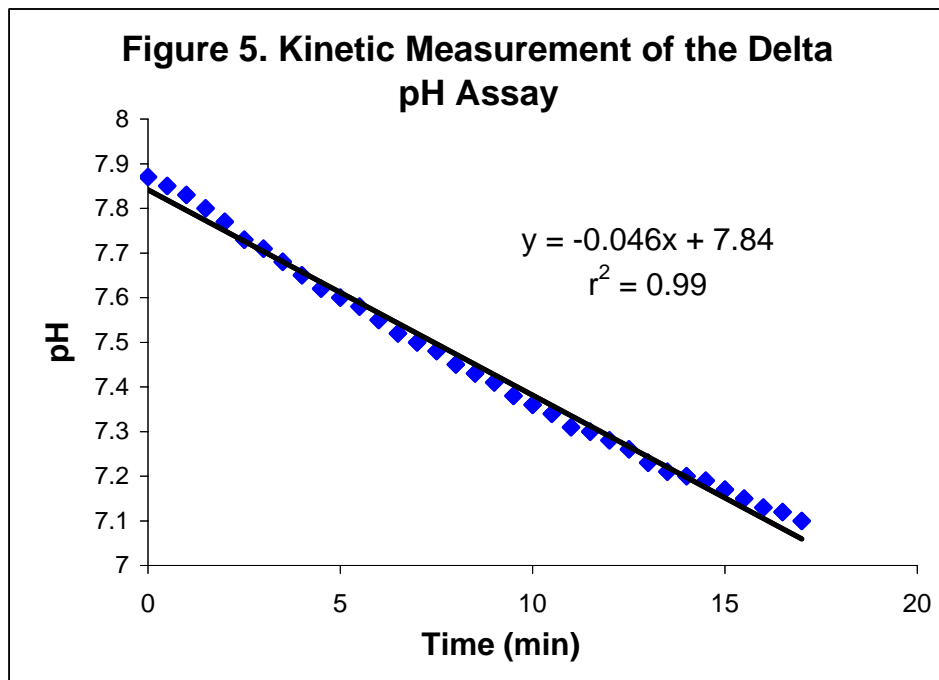
Delta pH activities of 991 DOD personnel from the CHPPM database converted to Ellman units using the interlaboratory conversion equation:

$$\text{Ellman} = 15.0(\text{delta pH/hr}) - 3.06$$



Delta pH activities of 991 DOD personnel from the CHPPM database converted to Ellman units using the DFP Inhibition conversion equation:

$$\text{Ellman} = 10.53(\text{delta pH/hr}) + 0.13$$



Change in pH during delta pH assay of a human RBC sample.